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Methodology article

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Effects of fish oil and dietary antioxidant supplementation on bone health

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Abstract

Background: Lambs or sheep are considered an excellent animal model for humans owing to advantages related to bone anatomy, formation, biomechanical characteristics; bone strength; and absorption of minerals and vitamins. Moreover, bone healing in many animal species is faster than that in humans, whereas bone healing, turnover, and remodelling in sheep and humans are comparable. In this context, it would be interesting to examine the effects of bioactive components of diet (including Se and carnosic acid) on bone mineralisation and strength, since these are the most important indicators of bone status. This knowledge may be useful in the context of orthopaedic research, recovery after orthopaedic surgeries, and prevention of skeletal diseases in humans.

Results: The aim of the present study was to assess the effects partial replacement of rapeseed oil (RO) with fish oil (FO) combined with dietary supplementation of various antioxidants on the characteristics of lamb femur. Thirty male lambs were assigned to five dietary treatments and fed isoproteinous and isoenergetic diets for 35 days. The control diet was enriched with 3.0% RO, while the experimental diets were enriched either only with 2.0% RO and 1.0% FO or additionally with 0.1% carnosic acid, 0.1% carnosic acid and 0.35 ppm Se as selenised yeast, or 0.1% carnosic acid and 0.35 ppm Se as sodium selenite. After 35 days, the lambs were slaughtered, and the femur was dissected from the carcass of each animal and analysed for morphometric properties.

Conclusions: The present study indicated that dietary bioactive components may improve bone health by promoting bone mineralisation in lambs. Partial replacement of RO with FO combined with dietary supplementation of carnosic acid and organic Se improved the geometric, densitometric, and biomechanical properties of lamb femur.

Keywords: fish oil, antioxidant, diet supplementation, lamb femur

Background

In previous studies, dietary supplementation of n-3 polyunsaturated fatty acids (n-3 PUFA) improved the growth, meat fatty acid profile, fertility, and immunity of pigs [1,2], chicken [3], ruminants [4,5], and rabbits [6]. However, several of these studies used fats rich in n-3 PUFAs, primarily linseed oil [a source of α-linolenic acid (ALA, C18:3n-3)] or fish oil [FO; a source of docosahexaenoic acid (DHA, C22:6n-3)] but tested the benefits of n-3 PUFA supplementation of animal feed irrespective of the dietary source. In some studies, n-3 PUFA supplementation of diet improved the fatty acid profile as well as degree of bone mineralisation and bone strength in monogastric animals [2,7]. Thus, dietary supplementation of n-3 PUFA in monogastric animals is beneficial for both animals (by improving their welfare) and humans (by improving the composition of animal products, such as meat). Recently, much scientific efforts was put into the modification of fatty acid profile of ruminant tissues [5,8,9]. However, achieving benefits in this group of animals is rather difficult due to differences in the structure of the gastrointestinal tract. Therefore, studies on the modification of dietary fatty acid composition and its effects on ruminant tissues have been conducted using various feed supplements (e.g. oils or phytochemicals). Moreover, additional studies have been designed to reduce of bacterial lipolysis and subsequently supress biohydrogenation and isomerisation in rumen, mainly through decreasing the enzymatic isomerisation yield of linoleic acid (LA, C18:2n-6) or ALA via inhibition of ruminal bacterial isomerase activity [10,11,12].

Furthermore, Miezeliene et al. [13] proposed that Se regulates the key pathways of antioxidant defence mechanism in the body. In addition, Davis et al. [14] reported that Se is an essential constituent of selenoenzymes, which play pivotal roles in various physiological processes. Se is commonly added to animal diets in an inorganic (sodium selenite) or organic (e.g. selenised yeast) form; however, Gjerlaug-Enger et al. [15] and Rayman [16] demonstrated that the organic form shows greater bioavailability and is more effectively taken up by tissues. Some studies [17,18] indicated that partial replacement of rapeseed oil (RO) with FO [(rich in long-chain n-3 PUFA (LCPUFA)] combined with supplementation of carnosic acid and Se compounds (both organic and inorganic forms) affected rumen isomerisation and biohydrogenation, further decreasing the tissue concentration of undesirable saturated fatty acids (SFA) and increasing the tissue concentration of desirable unsaturated fatty acids (UFA), particularly LCPUFA.

Moreover, according to Rozbicka-Wieczorek et al. [9], such a dietary supplementation decreased oxidative processes in the animal body. Oxidative stress disrupts bone remodelling, consequently reducing bone mass and bone density and increasing bone susceptibility to

fractures [19]. In addition, some clinical studies [20,21] have shown that antioxidants play important roles in reducing inflammatory processes, which negatively affect bone turnover. Consistent with this, another study [22] in rabbits showed that the combined administration of sodium selenite with vitamins E and C (with antioxidant properties) was more effective in preventing structural alterations of bones than the use of vitamins alone.

However, the effects of partial replacement of RO with oils rich in LCPUFA, such as FO, combined with the supplementation of carnosic acid and organic or inorganic Se compounds on bone strength and mineralisation in animal models, especially those other than rodents, remain unknown. Increasing evidence indicates [23,24] that lambs or sheep can serve as an excellent large animal model for humans in studies on orthopaedic or dental defects, owing to advantages related to bone anatomy, formation, and biomechanical characteristics; ease of handling; and absorption of minerals and vitamins. Moreover, bone healing in many non-human animal species is faster than that in humans, whereas this rate is comparable in sheep and humans [25,26]. Additionally, sheep bones have been previously established as useful models for human bone turnover and remodelling [23]. In this context, it seems interesting to assess the effects of dietary supplementation of various oils combined with antioxidants (carnosic acid and organic or inorganic Se compounds) on bone characteristics in a sheep model. These analyses will advance our understanding of the association between dietary supplementation of various antioxidants and bone properties as well as the related metabolic processes. This information will also allow for better design and conduct of research using a lamb model (e.g. the role of natural dietary supplements in orthopaedics, recovery after orthopaedic procedures, and prevention of skeletal diseases).

To this end, we hypothesised that partial replacement of RO with FO combined with supplementation of various antioxidants (carnosic acid and Se compounds) in diets would improve the tissue profile of n-3 PUFA, mineralisation, geometry, and biomechanics of lamb femur. Therefore, the primary aim of the present study was to evaluate the effects of various dietary modifications on femur morphometry, cortical wall thickness (CWT), cross-sectional area (CSA), cortical index (CI), and strength in a lamb model.

2. Material and Methods

2.1. Ethics

All experimental procedures in this study were performed in accordance with the relevant national or local ethical guidelines and were approved by the III Local Ethics Committee on Animal Experimentation of Warsaw University of Life Sciences, SGGW,

Poland. According to the principles of the 3Rs (replacement, reduction, and refinement), the study and experiments were designed to minimise the number of animals whilst maintaining high statistical power.

2.2. Animal experiments

Material for research was obtained from an experiment evaluating the effects of dietary modification on the profile of biohydrogenation products, specifically conjugated fatty acids, in ruminal fluid and some tissues as well as microbiota [18]. The femur was collected, as this bone is commonly used in human osteoporosis and orthopaedic research. Since densitometric measurements are commonly used in human medicine, the present study focused mainly on the state of bone mineralisation owing to its great impact on bone strength.

The study was performed in 30 male Corriedale lambs. The animals were randomly stratified into five groups (n = 6 animals each) and individually kept in pens on rubber mats. The main experiment was performed following a 3-week initial period (change in body weight from ~25 to 30 kg). During this period, the lambs were fed only a basal diet (BD) containing 36.0% meadow hay and 64.0% concentrate with soybean meal (360 g), barley (165 g), wheat starch (90 g), and a mineral–vitamin mixture (25 g). When lambs reached the body weight of 30 kg, BD was supplemented with 3.0% RO (group C); RO (2.0%) + FO (1.0%) (group EI); RO (2.0%) + FO (1.0%) + carnosic acid (0.1%) (group EII); RO (2.0%) + FO (1.0%) + carnosic acid (0.1%) + organic Se (0.35 ppm selenised yeast) (group EIII); or RO (2.0%) + FO (1.0%) + carnosic acid (0.1%) + mineral Se (0.35 ppm sodium selenite) (group EIV). These feeding schemes were maintained for the following 35 days (until the lambs reached ~37.0 kg body weight).

Feeding schemes as well as feed chemical composition, energy content, and fatty acid concentration are presented in Tables 1-3. During both initial and experimental periods, animals had semi-*ad-libitum* access to diets (0.85 and 1.08 kg·day-1, respectively), offered twice a day (7.30 am and 4.00 pm) in equal amounts, and *ad-libitum* access to fresh water. The control and experimental diets were isoenergetic and isonitrogenous and constituted (including supplements) the following contents per kilogram dry matter, according to the feeding recommendations for ruminants [27]: 17.9 MJ gross energy, 202 g crude protein, 119 g crude fibre, and 51.7 g crude fat. RO and commercial odourless FO containing high amounts of LCPUFA were purchased from Agrosol (Pacanów, Poland), and carnosic acid was purchased from Hunan Geneham Biomedical Technology Ltd. (Hunan, China). Selenised yeast

(*Saccharomyces cerevisiae*) was purchased from Sel-Plex (Alltech In., USA), and sodium selenite was provided by Sigma-Aldrich (St. Louis, MO, USA).

At the end of the main experimental period (35 days), after 12 h of starvation, each lamb was anesthetised via intramuscular xylazine injection (2-4 mg·10 kg-1 body weight) and then slaughtered by exsanguination. Next, from each right half-carcass, the femur was dissected, cleaned of any remaining flesh, weighed, and frozen (-30°C) for subsequent analyses.

2.3. Determination of chemical composition

Dry matter, nitrogen, ash, crude fibre, and ether extract contents of the diets were determined using the standard methods 934.01, 984.13, 942.05, 978.10, and 920.39 of the Association of Official Analytical Chemists [28], respectively. Fatty acid content of the feeds and each diet component was determined by base- and acid-catalysed methylation, as described by Czauderna et al. [29], followed by quantification using capillary gas chromatography coupled with mass spectrometry (GC–MS), as described by Rozbicka-Wieczorek et al. [9] GCMS-QP2010 Plus EI (Shimadzu, Tokyo, Japan) equipped with a BPX70 fused silica column (120 m × 0.25 mm i.d. × 0.25 μm film thickness; Phenomenex, Torrance, CA, USA), a quadruple mass selective detector (Model 5973 N), and an injection port was used, with helium as the carrier gas. Fatty acid methyl esters (FAMEs) were identified by the comparison of electron ionisation spectra of standards (Sigma, St. Louis, MO, USA) and the NIST 2007 reference mass spectra (National Institute of Standard and Technology, Gaithersburg, MD, USA). All FAME analyses were based on total ion current chromatograms and/or selected ion monitoring chromatograms.

2.4. Densitometric measurements of the femur

Dual-energy X-ray absorptiometric scans of the femur were obtained using the XR-800TM (Norland Medical Systems, CooperSurgical, Fort Atkinson, WI, USA) densitometer scanner according to the manufacturer's protocol of scanning and analysis (research-scan type). A quality assurance test was performed every day to verify the stability of the system calibration (control scans). Moreover, the system was calibrated daily using the QC Phantom and QA Calibration Standard (Norland Medical Systems). Specimens for scanning were thawed at 23°C for 12 h prior to use. During scanning, the right femur was positioned horizontally, with the femoral head facing upwards and the condyles facing downwards, and then scanned from the distal to proximal end. All scans were performed in triplicate to avoid bone rotation, as inconsistencies in the orientation can hamper the accuracy of test results. To ensure consistency,

all scans were performed by the same operator. Bone mineral content (BMC) and bone mineral density (BMD) were recorded.

2.5. Three-point bending test of the femur

Following dual-energy X-ray absorptiometry, a three-point bending test was performed using a TA-HDi texture analyser (Stable Micro Systems Ltd.) to determine the biomechanical properties of the right femur, as described by Ferretti et al. [30]. The distance between bone supports was set at 40% of the femur length, and the measuring head loaded bone samples at the mid-shaft at a constant speed of 50 mm·min-1. Maximum strength (MS) and maximum elastic strength (MES) of the bone were determined.

2.6. Geometric measurements of the femur

Geometric properties of each femur were determined based on the measurements of horizontal and vertical diameters (both external and internal) using an electronic ruler after cutting the bone. CWT (mm), CSA (mm2), and CI (%) were determined using the following mathematical formulae:

$$Cortical\ wall\ thickness\ (CWT) = \frac{[(V + H) - (v + h)]}{4}$$

$$Cross - section\ area\ \ (CSA) = \frac{\pi \times [(H \times V) - (h \times v)]}{4}$$

$$Cortical\ index\ (CI) = \left(\left(\frac{H - h}{H} + \frac{V - v}{V}\right) \div 2\right) \times 100$$

where V is the vertical external diameter (mm); H is the horizontal external diameter (mm); v is the vertical internal diameter (mm); and h is the horizontal internal diameter (mm).

2.7. Statistical analysis

Statistical analyses were performed using Statistica (version 12, StatSoft, Tulsa, OK, USA). The examined bone characteristics in different groups are presented as mean values, with statistical errors pooled as standard error. Results were analysed with one-way ANOVA. When the *F* ratio was significant, Tukey test was used to determine differences between groups. Statistical significance was set at $P < 0.05$. With an α level of 0.05, the power established at

80%, and an effect size of 0.75, the required total sample size was 30 (i.e. n = 6 per group). The hypothesised effect size of 0.75 was established based on descriptive statistics of a previous study [2]. *Post hoc* calculations using the above-mentioned data indicated that the actual power achieved in this study was 81.4%.

3. Results

The experimental factors did not affect the growth rate of lambs. There were no significant differences in body weight across lamb groups (30.5 ± 0.5 and 37.3 ± 0.60 kg at the beginning and end of the main experiment, respectively).

Femur mass and length did not differ across groups (mean 135 g and 17.24 cm, respectively Table 4). Femur CWT and CSA did not differ between groups EIII and EI (mean 3.24 mm and 157 mm2, respectively); however, values in groups EIII (P = 0.0153) and EI (P = 0.0461) were greater than those in C, EII, and EIV (mean 3.13 mm and 149 mm2, respectively). The highest femur CI was recorded in group EIII (39.4%; P = 0.0315), followed by groups EI and EIV (mean 35.9%), while the lowest CI was recorded in groups C and EII (mean 35.0%). The highest femur BMC was recorded in group EIII (37.20 g), followed by groups C, EI, and EII (mean 34.9 g), while the lowest BMC was recorded in group EIV (33.9 g; P = 0.0003). The highest femur BMD was recorded in group EIII (0.768 g·cm-2), followed by groups EII, EI, and EIV (mean 0.716 g·cm-2), while the lowest BMD was recorded in group C (0.703 g·cm-2, P = 0.0001). However, BMD in groups EI and EIV did not significantly differ from values in groups C and EII. Femur MS was recorded in following order (P < 0.0002): group EIII (281 N) > EII and EI (mean 259 N) > C and EIV (mean 246 N). MES followed the same order as MS (P = 0.0006).

4. Discussion

In the present study, the energetic and nutritive value of diets as well as feed intake was equalised in all experimental groups. Thus, the diets offered to lambs in a particular group differed only in terms of the content of LCPUFA in fat source of supplements. Previous studies on the modification of fatty acid composition of diets were designed to reduce bacterial lipolysis and subsequent biohydrogenation and isomerisation in the rumen [10,12].

Lambs or sheep are considered an excellent animal model for humans [23,24], owing to advantages related to bone anatomy, formation, biomechanical characteristics; bone strength; and absorption of minerals and vitamins. Moreover, bone healing in many animal species is faster than that in humans, whereas bone healing [25,26], turnover, and remodelling [23] in

sheep and humans are comparable. In this context, it would be interesting to examine the effects of bioactive components of diet (including Se and carnosic acid) on bone mineralisation and strength, since these are the most important indicators of bone status. This knowledge may be useful in the context of orthopaedic research, recovery after orthopaedic surgeries, and prevention of skeletal diseases in humans.

Previous studies using porcine [2] and rats [31] models indicated that dietary supplementation of n-3 PUFAs improved bone mineralisation and strength. In the present study, we also found a positive effect of partial replacement of RO (a source of LA) with FO (a source of EPA and DHA) on the tested bone parameters. In the body, elongase and desaturase convert LA to arachidonic acid (AA) [32]. However, both EPA and AA are substrates for the production of eicosanoids, particularly prostaglandins (PGs). PGE2 is a product of n-6 PUFA, and PGE3 is a product of n-3 PUFA, and these PGs exert antagonistic effects. PGE2 exerts inflammatory effects, whereas PGE3 exerts anti-inflammatory effects. The inflammatory effects of PGE2 and other inflammatory cytokines (e.g. IL-1, IL-6, and TNF-α) suppress bone formation and enhance bone resorption [33], possibly through upregulating the osteoclast activator RANKL [34]. However, n-3 PUFA inhibit this reaction [35] and promote osteoblastogenesis in the bone marrow [36] and bone formation [37]. Interestingly, in a rats [31] and pigs [38] model, diets rich in ALA did not affect femur morphology, biomechanical properties, and bone mineral content and density compared with diets rich in LA or SFA. This can be explained by the mammalian fatty acid metabolism: limited desaturases are available in LCPUFA synthesis, leading to low endogenous EPA and DHA levels [32]. Elevated PGE3 synthesis also alters cell membrane structure and fluidity, which facilitates vitamin D permeation, thus playing a crucial role in the active transport of Ca across the cell membrane [39,40]. Therefore, if EPA and DHA contents of diet exceed AA content, less substrate is available for eicosanoid synthesis from AA. Thus, this fatty acid composition of diet positively affects bone health.

Carnosic acid is a major bioactive component of rosemary and has been reported to show antioxidant and anti-inflammatory properties [41,42]. Moreover, it enhanced bone formation and inhibited bone resorption [43]. Above cited authors hypothesised that although carnosic acid is degraded in the body, its inhibitory effects on the formation of multinucleated osteoclasts would support the osteoblastic differentiation. According to these authors inhibition of osteoclast formation is the key action of carnosic acid that improves bone health. Unfortunately, however, bone metabolism at the cellular level was not studied in the present study thus, we cannot prove this hypothesis. Regarding the effects of carnosic acid on bone mineralisation, our results indicated that the supplementation of lamb diets with this acid

269 improved bone mineral content and bone mineral density to similar extents as did the partial
270 replacement of RO with FO.

271 Se regulates the key pathways related to antioxidant defence mechanisms in all tissues
272 by controlling glutathione metabolism through major Se-containing antioxidant enzymes.
273 Moreover, Se is an essential nutrient, which plays pivotal roles in various physiological
274 processes as an essential constituent of nearly 25 selenoenzymes, in which it is present as the
275 selenoamino acid selenocysteine [44]. Se is commonly added to animal diets in an inorganic
276 form (sodium selenite). However, owing to low bioavailability [15], much of inorganic Se is
277 excreted from the body and is thus not effectively taken up by tissues, including bones [16].
278 Moreover, this form of Se may exert pro-oxidant and even toxic effects, particularly at high
279 levels [45,46]. Therefore, recently, interest in the organic form of Se has increased because of
280 its better absorption and greater biological effectiveness in pigs [47], broilers [48], beef cattle
281 [49], and laying hens [50]. In a study on rabbits Ebeid et al. [51] found that bioactive compounds
282 (e.g. organic Se) positively affect body metabolism. They hypothesised that the mechanism of
283 action of these bioactive compounds is based on the mitigation of oxidative stress, which
284 consequently protects UFA from peroxidation damage, and these UFA can then be effectively
285 taken up by soft tissues. Thus, similar effects are likely produced in bone, as evidenced by the
286 positive effects of n-3 PUFA on bone parameters in a previous study on rats [31].

287 In the present study, we found a positive effect of simultaneous administration of a
288 mixture of RO (a source of n-6 PUFA with pro-oxidative properties) and FO (a source of n-3
289 LCPUFA with antioxidative properties) with carnosic acid and organic Se on the tested bone
290 parameters: geometrical, biomechanical, and densitometric parameters of femur in lambs that
291 received this diet were improved compared with those of lambs that received other diets. The
292 mixture of such bioactive compounds likely acted synergistically on the metabolism of fatty
293 acids in the rumen as well as on their accumulation in the tissues of lambs, including the
294 skeleton.

295 To the best of our knowledge, no study in the literature has explored the effects of
296 simultaneous administration of carnosic acid and organic Se with a mixture of RO and FO on
297 skeletal metabolism, bone mineralisation, and bone strength in ruminants. The present study
298 demonstrated that a diet containing a mixture of RO and FO enriched with carnosic acid and
299 organic Se improved the tested parameters of lamb femur. Based on previous reports [52], we
300 believe that this effect was caused by the greater efficacy of organic Se than that of inorganic
301 Se in increasing the content of this element in tissues. Inorganic Se is retained in the body for a
302 short period, and only small amount of Se is incorporated into selenoproteins, while majority is

303 excreted through urine. Studies on rabbits [53], bulls [54], and pigs [55] have demonstrated
304 greater accumulation of organic Se in meat tissues. Other studies have also shown that Se is an
305 essential dietary nutrient, which plays vital roles in bone health through promoting bone
306 formation during bone turnover [15,25,56] and reducing the risk of bone fracture in both
307 animals [15,24] and humans [56,57]. This can be explained by the fact that Se regulates a major
308 part of the antioxidant defence mechanism by controlling glutathione metabolism through the
309 key Se-containing antioxidant enzymes glutathione peroxidase and thioredoxin reductase
310 [13,58]. In turn, glutathione peroxidase protects the integrity of unsaturated bonds in membrane
311 phospholipids by preventing damage caused by free radicals, which can initiate lipid
312 peroxidation [58]. Therefore, these properties of Se (and other antioxidants) have been believed
313 to protect body cells from the imbalance between oxidants and antioxidants and subsequent
314 oxidative stress, which is considered to be the primary pathogenesis of skeletal disorders (e.g.
315 low bone mineral density or decreased in bone mass) that make bones more prone to fractures
316 [20,59].
317 In summary, a large part of function of maintaining the growth and health of bones is
318 attributed to the balance between osteoclast and osteoblast activities, which regulate bone
319 remodelling. Recent evidence has shown that bioactive substances (e.g. LCPUFA, carnosic
320 acid, and organic Se), including those contained in the diet, play crucial roles in maintaining
321 normal bone remodelling processes and protecting bone health. They prevent and/or relieve
322 oxidative stress, inflammation, and changes in cell membrane structure and fluidity, thereby
323 inhibiting osteocyte apoptosis and mitigating osteoclast activity to ultimately increase
324 osteoblast activity and osteogenesis. Thus, such compounds may be used as dietary supplements
325 for maintaining bone health, preventing skeletal diseases, and facilitating recovery following
326 orthopaedic procedures.
327
328 **Conclusion**
329 The present study indicated that dietary bioactive components may improve bone health
330 by promoting bone mineralisation in lambs. Partial replacement of RO with FO combined with
331 dietary supplementation of carnosic acid and organic Se improved the geometric, densitometric,
332 and biomechanical properties of lamb femur.
333

Table 1. Scheme of the study (diet and supplements consumption by particular group of lambs during main experiment)

Group/ diet	Preliminary (3 weeks)	Main experiment (5 weeks)					
		Diet	Supplement				
	Diet		RO (%)	FO (%)	Carnosic acid (%)	SeY (ppm)	Na_2SeO_3 (ppm)
C	BD	BD	3.0	-	-	-	-
E_I	BD	BD	2.0	1.0	-	-	-
E_{II}	BD	BD	2.0	1.0	0.1		
E_{III}	BD	BD	2.0	1.0	0.1	0.35	-
E_{IV}	BD	BD	2.0	1.0	0.1	-	0.35

C – animals fed basal diet + 3.0% rapeseed oil; E_I - animals fed basal diet + 2.0% rapeseed oil + 1.0 % fish oil; E_{II} – animals fed basal diet + 2.0% rapeseed oil + 1.0% fish oil + 0.1% carnosic acid; E_{III} – animals fed basal diet + 2.0% rapeseed oil + 1.0% fish oil + 0.1% carnosic acid + 0.35ppm SeY; E_{IV} - animals fed basal diet + 2.0% rapeseed oil + 1.0% fish oil + 0.1% carnosic acid + 0.35ppm Na_2SeO_3; SeY - selenized yeast (Saccharomyces cerevisiae); RO – rapeseed oil; FO – fish oil; BD – basal diet (1kg include: meadow hay – 360 g, concentrate consisting: soybean meal – 360 g, barley – 165 g, wheat starch – 90 g and mineral-vitamin mixture – 25 g); Mineral - Vitamins mixture in the diet provided by POLFAMIX OK (Grodzisk Mazowiecki, Poland), supplied per kg of diet: g: Ca 285, P 16, Na 56, Fe as sulphate 1, Cu as sulphate 0.5, Mn as sulphate 5.8, Zn as sulphate 7.5; mg: Co as carbonate 42, I as iodate 10, Se as selenite 6; and IU: vit. A 500 000, vit. D3 125 000, vit. E as α-tocopherol 25 000

350 Table 2. Chemical composition and energy content in the ingredients of diets

Item	Meadow hay	Concentrate		
		Barley meal	Soybean meal	Wheat starch
Dry matter (DM), %	88.4	87.6	89.7	87.3
In DM, %				
crude protein	9.50	9.94	41.8	0.90
crude fibre	27.3	2.87	4.34	-
crude fat	3.40	2.50	2.25	0.09
ash	4.85	1.84	6.16	0.12
Gross energy, MJ/kg DM	17.1	16.3	17.8	16.7

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352
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Table 3. Long chain fatty acid concentration (g/kg) in the ingredients and diets fed to animals.

Fatty acids	Ingredients				Period of the study		
					Preliminary	Main experiment (diet/group)	
	Concentrate	Meadow hay	RO	FO	BD	C	E_I, E_{II}, E_{III}, E_{IV}
C18:2 n-6 (LA)	29.2	13.1	282.0	115.0	20.0	28.5	26.84
C18:3 n-3 (ALA)	1.01	4.18	38.5	21.0	2.04	3.19	3.02
C20:5 n-3 (EPA)	nd	nd	nd	6.79	nd	nd	0.07
C22:5 n-3 (DPA)	nd	nd	nd	1.56	nd	nd	0.02
C22:6 n-3 (DHA)	nd	nd	nd	26.6	nd	nd	0.27

C – animals fed basal diet + 3.0% rapeseed oil; E_I - animals fed basal diet + 2.0% rapeseed oil + 1.0 % fish oil; E_{II} – animals fed basal diet + 2.0% rapeseed oil + 1.0% fish oil + 0.1% carnosic acid; E_{III} – animals fed basal diet + 2.0% rapeseed oil + 1.0% fish oil + 0.1% carnosic acid + 0.35ppm SeY; E_{IV} - animals fed basal diet + 2.0% rapeseed oil + 1.0% fish oil + 0.1% carnosic acid + 0.35ppm Na_2SeO_3; SeY - selenized yeast (Saccharomyces cerevisiae); RO – rapeseed oil; FO – fish oil; BD – basal diet (1 kg include: meadow hay - 360g, concentrate consisting of: soybean meal - 360g, barley - 165g, wheat starch - 90g and mineral-vitamin mixture - 25g); Mineral-Vitamins mixture in the diet provided by POLFAMIX OK (Grodzisk Mazowiecki, Poland), supplied per kg of diet: g: Ca 285, P 16, Na 56, Fe as sulphate 1, Cu as sulphate 0.5, Mn as sulphate 5.8, Zn as sulphate 7.5; mg: Co as carbonate 42, I as iodate 10, Se as selenite 6; and IU: vit. A 500 000, vit. D3 125 000, vit. E as α-tocopherol 25 000; nd – not determined

Table 4. Femur morphometric, geometric, densitometric and biomechanical properties at the end of the study in lambs

item	Group/diet					SE	P - Value
	C	E_I	E_{II}	E_{III}	E_{IV}		
Mass, g	136	137	135	135	132	4.09	0.9491
Length, cm	17.3	17.4	17.2	17.4	16.9	0.20	0.3572
CWT, mm	3.16^{a}	3.21^{b}	3.11^{a}	3.26^{b}	3.13^{a}	0.104	0.0153
CSA, mm^2	149^{a}	154^{b}	149^{a}	160^{b}	148^{a}	4.819	0.0461
CI, %	34.7^{a}	35.9^{b}	35.3^{a}	39.4^{c}	36.0^{b}	1.281	0.0315
BMC, g	34.3^{B}	35.1^{B}	35.4^{B}	37.2^{C}	33.9^{A}	0.618	0.0003
BMD, g/cm^2	0.703^{A}	0.713^{AB}	0.725^{B}	0.768^{C}	0.710^{AB}	0.009	0.0001
MES, N	182^{A}	190^{B}	188^{B}	216^{C}	183^{A}	5.101	0.0006
MS, N	245^{A}	261^{B}	257^{B}	281^{C}	247^{A}	5.509	0.0002

C – animals fed basal diet + 3.0% rapeseed oil; E_I - animals fed basal diet + 2.0% rapeseed oil + 1.0 % fish oil; E_{II} – animals : basal diet + 2.0% rapeseed oil + 1.0% fish oil + 0.1% carnosic acid; E_{III} – animals fed basal diet + 2.0% rapeseed oil + 1.0% fish oil + 0.1% carnosic acid + 0.35ppm SeY; E_{IV} - animals fed basal diet + 2.0% rapeseed oil + 1.0% fish oil + 0.1% carnosic acid + 0.35ppm Na_2SeO_3; SeY - selenized yeast (Saccharomyces cerevisiae); CWT – cortical wall thickness; CSA – cross sectional area; CI – cortical index; BMC – bone mineral content; BMD – bone mineral density; MES – maximum elastic strength; MS – maximum strength;; SE – pooled standard error of mean; ^{A,B,C} mean values within a rows with unlike superscript letters were significantly different at $P<0.01$; ^{a,b,c} mean values within a rows with unlike superscript letters were significantly different at $P<0.05$.

Highlights

Supplementation a diet with long-chain n-3 fatty acids improves heath of the bones.

Addition of carnosine acid to a diet containing long-chain fatty acids does not improve bone health.

Long-chain n-3 fatty acids, carnosic acid and selenised yeast shows a synergistic effect and improve bone health in the greatest degree.

Declarations:

Ethics approval and consent to participate: Authors declare that they have no conflict of interest. This article does not contain any studies with human participants performed by any of the authors.

Consent for publication: All authors have read and agreed to the published version of the manuscript.

Availability of data and material: data and material available at any time upon request of reviewers or potential users

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According to 3R (Replacement, Reduction, Refinement) ethical principle the design of the study and experimental techniques used through the analysis allowed to minimize the number of animals with maintaining high statistical precision. There is no way to completely replace live animals with another research model. Thus, lambs have been chosen as a model for humans in the area of orthopedic, bone defects filling due to factors strictly related to bone anatomy, bone turnover and remodeling, biomechanical features, absorption of minerals and vitamins. Moreover, in contrast to other animal species sheep/lambs have a bone healing rate close to humans.

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Conflict of Interest

None.

Statement

The amount and source of fat and antioxidants used in animal study could reasonably be expected to be achieved in the human population, e.g., in food products, among other as a component in vegetable salad, as a cooking oil.

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